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Treatment of cancer with chemotherapy and radiation therapy has severe side effects that damage healthy proliferating cells such as hematopoietic precursors, hair follicle, cells and the epithelial lining of the intestine. These side effects often limit the doses of chemotherapy administered, allowing tumor cells to gain growth advantage by escaping treatment and developing drug resistance. Since cancer therapy targets proliferating cells and tissues, all cells that proliferate, whether normal or tumor are affected by the treatment. If however the normal dividing cells in the body were to stop proliferating reversibly, the toxic effects of chemotherapy would potentially be diminished. Here, we introduce a novel therapeutic strategy to selectively target cancer cells, while leaving normal proliferating cells intact by taking advantage of differences in cell cycle regulation between normal and tumor cells. The approach taken for this "protection" strategy involves two steps: First, the normal proliferating cells are blocked in the G0/G1 phase of the cell cycle by pre-treatment with cytostatic, non-lethal agents. Tumor cells will not respond to these agents, because they have lost the G0/G1 checkpoint; they will continue to proliferate. Next, both normal and tumor cells are treated with conventional chemotherapeutic agents which will specifically kill proliferating tumor cells. Normal cells are protected because of the G0/G1 mediated reversible arrest achieved in the first step. Our goal for this one year concept award is to examine the feasibility of our hypothesis against drug resistant tumor cells in culture and to initiate a pilot study in vivo to examine the pre-clinical applicability of our hypothesis in mice.

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Introduction:

The overall purpose of this 1 year study was to initiate an in vivo study in mice examining the feasibility of a treatment strategy where normal cells are protected from the toxic effects of chemotherapeutic agents, by reversibly arresting them in the G1 phase of the cell cycle.

Body:

Treatment of cancer with chemotherapy and radiation therapy has severe side effects that damage healthy proliferating cells such as hematopoietic precursors, hair follicle, cells and the epithelial lining of the intestine. These side effects often limit the doses of chemotherapy administered, allowing tumor cells to gain growth advantage by escaping treatment and developing drug resistance. Since cancer therapy targets proliferating cells and tissues, all cells that proliferate, whether normal or tumor are affected by the treatment. If however the normal dividing cells in the body were to stop proliferating reversibly, the toxic effects of chemotherapy would potentially be diminished (1-5). In this one year concept award, we proposed to investigate the feasibility of a novel therapeutic strategy to selectively target cancer cells, while leaving normal proliferating cells intact-in vivo. This strategy of protection from chemotherapy is based on the genotypic differences between normal and tumor cells that govern cell cycle regulation (6). Unlike normal cells, which are controlled by tight cell cycle checkpoint regulation, tumor cells have a deregulated cell cycle, which is responsible for their continued and unabated proliferation (7). Our treatment strategy takes advantage of this difference in cell cycle regulation between normal and tumor cells, maintaining the normal cells in a state of reversible G0/G1 arrest while selectively killing the tumor cells with cytotoxic chemotherapeutic agents.

To explore the feasibility of cellular protection through reversible cell cycle arrest, we target the pRb (retinoblastoma) pathway, which governs the transition from G0/G1 to S, or the restriction point (8). pRb is a major tumor suppressor that is frequently inactivated in human cancer. Furthermore, alterations in pRb are linked to poor prognosis, tumor progression, and decreased sensitivity to chemotherapeutic agents (9-13). As an important tumor suppressor, pRb is involved in controlling the progression through G0/G1. The hypo-phosphorylated pRb serves as a tumor suppressor by binding to and inhibiting cellular proteins such as E2F-DP heterodimeric transcription factors. The activity of E2F is required for the transactivation of many genes essential for DNA replication, and is necessary for the cells to traverse from G0/G1 to S phase (8, 14-16). Sequential phosphorylation of pRb throughout the cell cycle by different cyclin/cyclin dependent kinase (CDK) complexes dissociates the pRb-E2F complexes and allows free E2F to transactivate genes that promote entry into S phase. Several studies have provided genetic and biochemical evidence that mutations in either the RB gene itself or in genes whose products influence its phosphorylation state (i.e cyclin D1, CDK4, CDK6, E2F), render the functional inactivation of pRb and contribute to tumor progression (4, 10, 17). The protection strategy described here takes advantage of the deregulated pRb pathway in tumor cells to protect normal cells against the toxic effects of chemotherapeutic agents.

Hypothesis/Rationale/Purpose:

Our hypothesis for this concept award is that by taking advantage of differences in cell cycle regulation between normal and tumor cells we can apply a novel therapeutic strategy to selectively target cancer cells, while leaving normal proliferating cells intact. The approach taken for this "protection" strategy involves two steps: First, the normal proliferating cells are blocked

in the G0/G1 phase of the cell cycle by pre-treatment with cytostatic, non-lethal agents. Tumor cells will not respond to these agents, because they have lost the G0/G1 checkpoint; they will continue to proliferate. Next, both normal and tumor cells are treated with conventional chemotherapeutic agents which will specifically kill proliferating tumor cells. Normal cells are protected because of the G0/G1 mediated arrest achieved in the first step. We have already successfully applied this protection strategy in vitro to protect normal mammary epithelial cells and normal human lymphocytes (stimulated) against the toxic effects of chemotherapeutic agents by pre-treating them with very low concentrations of Staurosporine (ST) or UCN-01 (i.e. cytostatic agents). Our results show that normal proliferating mammary epithelial cells or dividing lymphocytes can withstand otherwise lethal doses of chemotherapy (i.e. Doxorubicin and Camptothecin) if they are pretreated with ST or UCN-01, which arrests them in G0/G1 prior to administration of chemotherapy. Tumor cells are refractory to low concentrations of ST or UCN-01 and do not arrest in G1 due to defective G1 checkpoints. The G1 checkpoint targeted by low concentrations of ST or UCN-01 in normal cells is the pRb pathway which is tightly regulated in normal cells and defective in most tumors. Our goal for this concept Award is to examine the pre-clinical feasibility of our hypothesis in vivo (i.e. in mice).

Specifically, our objective was to examine the efficacy of the protection strategy in mice.

Results/Key Research Accomplishments:

During the past year we were successful in initiating our prospective analysis and providing initial results of the feasibility of our protection strategy. We used UCN-01 as the agent to arrest the normal rapidly proliferating epithelial lining of the intestine in the G1 phase of the cell cycle.

UCN-01 Inhibition of normal hyperproliferative cells in mice We performed initial in vivo dose response studies with UCN-01 in nude mice using the normal rapidly dividing intestinal epithelial cells as the normal tissue end point (Fig 1). We performed four sets of experiments (6 mice per dose of UCN-01) to determine the control kinetic parameters of jejunal mucosal cells and the perturbations of those kinetics by UCN-01. Mice were injected i.m. or i.p. with a single dose of UCN-01 (0, 2.5, or 10 mg/kg) followed 48 hours later by in vivo BrdUrd labeling at periods of 20 minutes or 6 hours prior to sacrifice of mice. Tissues harvested and processed as described below (18-25).

Methods for estimation of dynamic cell cycle kinetic parameters. The basis of these techniques is that cells in S-phase can be selectively labeled by administration of a nontoxic level of bromodeoxyuridine (BrdUrd). The cells that incorporate BrdUrd continue to progress through the cell cycle and may be sampled at a known time later. The sample, fixed in ethanol, may be processed to produce a suspension of single nuclei by enzymatic digestion with e.g., pepsin. The nuclei are analyzed simultaneously for BrdUrd and DNA content by flow cytometry. The BrdUrd-labeled nuclei are selectively stained by a monoclonal antibody to BrdUrd using a fluorescein isothiocyanate-conjugated (FITC, green fluorescing) second antibody technique. All the nuclei are also stained with PI, which fluoresces red at an intensity proportional to their DNA content, thereby simultaneously defining a reference standard for relative cell ages. At the time of labeling the BrdUrd-labeled cells are assumed to be completely and exclusively in the S-phase, with all unlabeled cells in the G₁ and G₂+M phases of the cell cycle. In the interval between the administration of BrdUrd and sampling, the cycling cells progress unperturbed to subsequent phases of the cell cycle. In particular, the

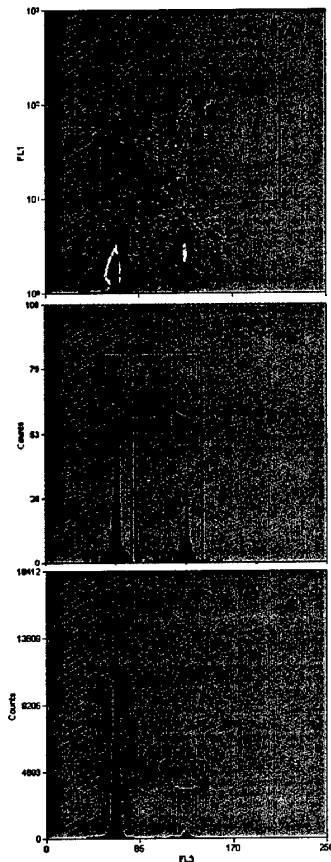
BrdUrd-labeled cells progress through S, G₂+M and, subsequently, into the next generation's G₁. These observations were the basis for the original method for calculation of the duration of DNA synthesis, T_S, and the potential doubling time, T_{pot}, from a single biopsy sample.

In vivo labeling and tissue fixation. Mice are pulse labeled with with 60 mg/kg body weight BrdUrd in PBS as an i.p. injection. Following sacrifice at the appropriate time later the jejunum is fixed in 65% ethanol in PBS at 4°C overnight before staining.

Staining procedure for jejunal mucosal cells. Following fixation, 2.5 inch lengths of jejunum are placed on a microscope slide, slit longitudinally and the mucosal surface gently scraped 5 times with the edge of another glass slide held at a 45° angle. The tissue so collected is processed to isolated nuclei by incubating for 60 minutes in a shaking water bath at 37°C in 5.0 ml pepsin (EM Sciences, Cherry Hill, NJ, 0.04% in 0.1N HCl). DNA is denatured with 3.0 ml of 2N HCl (1.5 ml per 2×10^6 nuclei) for 20 minutes at 37°C. After neutralization with 0.1M sodium borate nuclei are labeled with an indirect FITC-conjugated dual antibody procedure before resuspending in propidium iodide (PI, 10 mg/ml in PBTB) for a final concentration of 1×10^6 nuclei per ml. Samples are stored overnight at 4°C in the dark and run on the flow cytometer the next day .

Flow cytometry of labeled samples. Bivariate distributions of BrdUrd content (FITC) vs. DNA content (PI) are measured using a specially configured Epics 752 flow cytometer (Coulter Corp., Hialeah, FL) and 32 bit data acquisition electronics (Cicero, Cytomation, Fort Collins CO).

For these initial studies we labeled mice with 60mg/kg BrdUrd in PBS as an i.p. injection. At 20 minutes and 6 hours post BrdUrd injection, mice were sacrificed and the jejunum fixed overnight. The jejunal mucosal cells were collected, nuclei isolated, and labeled with FITC-conjugated dual antibody for BrdUrd and DNA content (propidium iodide) for 24 hours and then



subjected to flow cytometry (Fig 1). Using the analysis represented in Fig 1, our results revealed that the control values of BrdUrd in mice treated with DMSO alone (UCN-01 diluant) has a net cell production rate of 3.6% per day. After i.m. administration, however, of 10mg/kg UCN-01 the net cell production rate were halved to 1.7% per day. Additionally the fraction $f_{ld}(t)$ of BrdUrd-labeled divided cells at 6 hours was also reduced by a factor of 2 to 0.63% (Fig 1A). These observations infer a twofold prolongation of the duration of G1 as a result of a G1 block by UCN-01. Delivery of UCN-01 via the i.p route did not significantly change these kinetic parameter values (data not shown).

Figure 1. Bivariate DNA vs BrdUrd (linear integral red, FL3 - versus log green, FL1 - fluorescence) histograms 6 hours after a pulse label. The DNA vs. BrdUrd data for murine jejunal mucosa 2 days after 10 mg/kg UCN01 i.m. is presented. The populations from which kinetic parameters may be calculated are indicated. The animal was sacrificed 6 hours after a single i.p. injection of 60 mg/kg body weight BrdUrd. BrdUrd-labeled cells that remain undivided (f^u) and that have divided (f^d) in the 6-hour period after labeling can clearly be seen in

panel A. Figure 1, panel B shows the projection of BrdUrd-labeled cells from which the relative movement (RM^{lu}) of the BrdUrd-labeled cells that remained undivided at the time of sampling may be measured and hence the durations of S- and G_2+M phases calculated. Panel C of Figure 1 shows the univariate DNA profile (integral red fluorescence). The G_1 and G_2M peaks are indicated

Conclusion/Reportable Outcome:

During the one-year study, we have accomplished the goal set forth by our proposal. We have examined the feasibility of using a cytostatic agent in vivo to arrest the normal rapidly proliferating cells of the intestine in G_1 . This complex in vivo assay is now functional in our laboratory and we are now in a position to examine the ability of other, more clinically relevant, cytostatic agent in vivo.

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